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Synergistic enhancement of histamine release from rat peritoneal mast cells by the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate is not reflected by corresponding changes in phospholipid turnover

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In an attempt to elucidate further the relationship between changes in phospholipid metabolism in, and histamine secretion from, purified rat peritoneal mast cells, the effects of the phorbol diester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) on these responses in stimulated and unstimulated cells was investigated. TPA caused a dose-dependent increase in the incorporation of $^{32}PO_4^{3-}$ into the mast cell phospholipids; phosphatidic acid (PA) and phosphatidylcholine (PC), but not phosphatidylinositol (PI). TPA synergistically enhanced histamine release from cells stimulated by anti-immunoglobulin E (IgE) and the calcium ionophore A23187, reducing its ED_{50} from 150 nM to 40 nM, but did not alter histamine release from cells stimulated by compound 48/80. The effect of TPA on the changes in $^{32}PO_4^{3-}$ incorporation into phospholipids associated with the above secretagogues did not, however, correlate well with the observed effects on histamine secretion induced by the same secretagogues. These observations are discussed in relation to the known effects of phorbol esters upon both secretory processes and phospholipid metabolism in other tissues.

Introduction

The biochemical mechanisms by which tumour-promoting phorbol esters, such as TPA, modulate cellular proliferation and secretory functions have been partially characterised. TPA has been shown to be a potent activator of protein kinase C [1], which, together with raised intracellular Ca^{2+} levels caused by hydrolysis of phosphatidylinositol 4,5-bisphosphate, is a key event in Ca^{2+}-activated secretion [2]. TPA has been shown to modify phospholipid metabolism in many tissues (most commonly by causing increases in PA and PC turnover [3–5]). TPA has also been shown to potentiate Ca^{2+}-mediated secretory and mitogenic responses in many tissues stimulated with the Ca^{2+} ionophore A23187, e.g., superoxide release from neutrophils [6], insulin secretion from pancreatic islets [7] and serotonin release from platelets [8,9]. In particular, the synergistic action of TPA with A23187 and immunological stimuli has been demonstrated in polymorphonuclear

† Deceased.

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Abbreviations: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PI, phosphatidylinositol; PC, phosphatidylcholine; PA, phosphatidic acid; DMSO, dimethyl sulphoxide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; HTB, Hepes-buffered Tyrode solution. TLC, thin-layer chromatography; LDH, lactate dehydrogenase; PS, phosphatidylserine.

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leucocytes [10] and rat peritoneal mast cells [11,12]. The various histamine-releasing stimuli have also been shown to cause dose-related increases in $^{32}PO_4^{3-}$ incorporation into PI, PC and PA [13,14]. The present paper presents evidence to suggest first that TPA causes dose-related changes in phospholipid turnover in mast cells, most likely by a mechanism distinct from that employed by histamine-releasing secretagogues, and second that the effects of TPA upon histamine secretion induced by these agents does not correlate with the observed effects upon phospholipid metabolism. The results are discussed with reference to possible mechanisms of exocytosis in the mast cells.

Materials and Methods

Carrier-free [^{32}P]orthophosphate (10 mCi/ml) was supplied by Amersham International, plc. Compound 48/80 and TPA were supplied by Sigma. The TPA was diluted from a stock solution of 1 mg/ml in dimethyl sulphoxide (DMSO). The calcium ionophore A23187 was supplied by Boehringer, Mannheim, F.R.G. All other reagents were of AnalaR grade. Wistar rats were supplied by Bantin and Kingman, Hull, U.K.

Hepes-buffered Tyrode solution (HTB) (pH 7.4) comprising NaCl (137 mM)/KCl (2.7 mM)/ NaH_2PO_4 (0.4 mM)/$CaCl_2$ (1 mM)/$MgCl_2$ (0.5 mM)/Hepes (10 mM)/glucose (5.6 mM)/gelatine (1 mg/ml) was used for all procedures in which the rat mast cells were used.

Peritoneal mast cell suspensions were isolated from male rats (200–350 g body weight) by the method of Cooper and Stanworth [15]. After washing and removal of any erythrocyte contamination by resuspension of the cell pellet in hypotonic HTB, mast cells were purified by isopycnic centrifugation through Percoll (Pharmacia). A sample (1 ml) of a crude suspension of peritoneal cells in HTB (2–5% mast cells), containing approx. $1 \cdot 10^6$ mast cells, was layered on to 3 ml of 65% (v/v) Percoll in the same medium. After centrifugation for 15 min at $490 \times g$ at room temperature, mast cells, at a purity of 85–95% and 99% viability (determined by Trypan blue exclusion), were recovered as a pellet, after removal of the supernatant and cells remaining at the interface by aspiration.

Mast cells were labelled with $^{32}PO_4^{3-}$ as described by Kennerly et al. [13]. The mast cells were resuspended in phosphate-free HTB for 1 h prior to labelling and were washed frequently over that period with phosphate-free HTB. The cells were then resuspended to a density of $(1–2) \cdot 10^6$ cells/ml in phosphate-free HTB and carrier-free $^{32}PO_4^{3-}$ was added to a final concentration of 200 μCi/ml. The labelled suspension was incubated for 5 min at 37°C and 200 μl aliquots were removed and then added to tubes containing 50 μl of the same medium containing stimulating agent. Phosphate-free HTB alone served as a control. Incubations were continued for a further 15 min at 37°C and then terminated by addition of 2 ml ice-cold HTB, followed by centrifugation and washing. Phospholipids were extracted by the modification of the method of Bligh and Dyer [16] described by Kennerly et al. [13]. At this stage, 0.25 μmol of a carrier phospholipid mixture isolated from rat liver by the method of Radin [17] was added to improve extraction of the mast cell phospholipids. Commercial PI and PA (5–10 μg of each) (Lipid Products) was also added to aid visualisation by TLC.

Labelled phospholipids were separated by two-dimensional TLC on 20×20 cm silica gel 60 pre-coated plates (E. Merck, Darmstadt) using the following solvent systems in sequence: (I) chloroform/methanol/15 M ammonia (29% w/v), 65 : 35 : 5 (v/v); (II) chloroform/methanol/acetone/acetic acid/water, 50 : 20 : 10 : 10 : 5 (v/v).

Phospholipids were visualised by exposure of the plates to iodine vapour. Stained spots were scraped into scintillation vials and 5 ml of Fisofluor scintillation fluid (was added) (Fisons). Radioactivity in the samples was determined by scintillation spectrometry.

The identity of labelled mast cell phospholipids was confirmed by co-migration of label with authentic standards in other TLC systems and by autoradiography on Kodak XAR7 film.

Histamine release from parallel unlabelled incubations that had otherwise been treated in the same way as labelled preparations, was determined by the method of Shore et al. [18], but automated as described by Evans et al. [19]. Histamine release was expressed as a percentage

of total cellular histamine released from cell samples lysed in perchloric acid (0.4 M).

Lactate dehydrogenase (LDH) release was measured using an automated continuous-flow spectrophotometric method described by Riches and Stanworth [20]. LDH release was expressed as a percentage of total cellular LDH in lysates derived from Triton X-100-treated cell samples.

Increases in $^{32}PO_4^{3-}$ labelling of individual mast cell phospholipids were expressed as a percentage of dpm incorporated into unstimulated cells to compensate for quantitative variations in incorporation of radioactivity in different experiments.

Results

The effect of TPA on the incorporation of $^{32}PO_4^{3-}$ into phospholipids and histamine release

In the presence of TPA (10 ng/ml), mast cells released histamine linearly over a time course of 1 h; thereafter, the rate of release decreased. Maximal histamine release was 63.5% of total cellular histamine (Fig. 1). Over a period of 15 min, between 10% and 20% of cellular histamine was released by TPA. Since Heiman and Crews [12] did not observe any histamine release by the action of TPA alone on mast cells, it was essential to determine whether the observations made here were due to a direct effect of TPA upon the cell membrane. The release of LDH, an enzyme not normally released by intact cells, was therefore measured and it can be seen from Fig. 1 that TPA caused a significant release of LDH in parallel with histamine release. It is unlikely that the DMSO, used to dissolve the TPA, was the cause of cytotoxicity, since at the concentration of TPA used in this study (10 ng/ml), DMSO was diluted

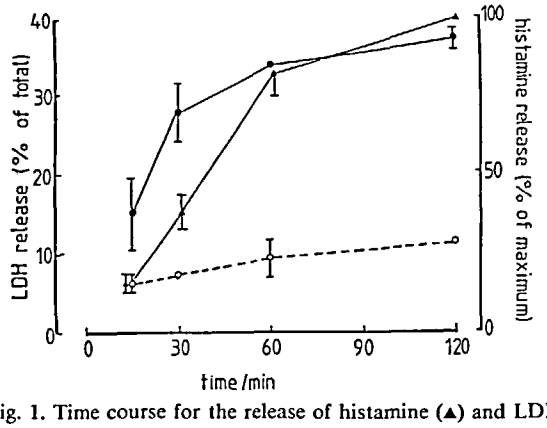

Fig. 1. Time course for the release of histamine (▲) and LDH (●) from mast cells in the presence of TPA (10 ng/ml). LDH release in the absence of TPA (○). Results are from a typical experiment performed in triplicate.

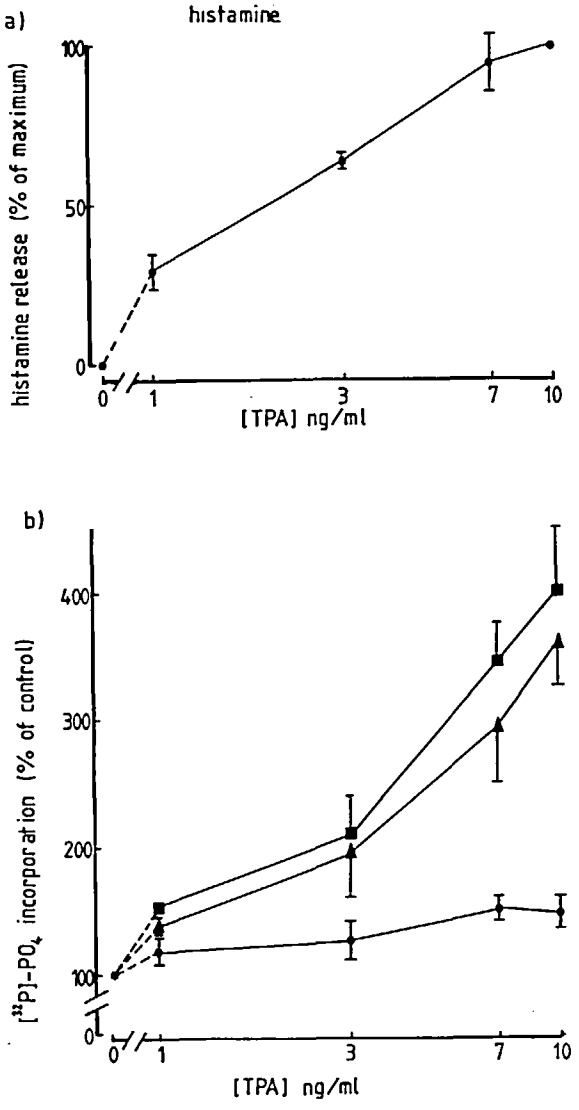

Fig. 2. Dose-related effects of TPA on (a) histamine release and (b) $^{32}PO_4^{3-}$ incorporation into PI (●), PC (■) or PA (▲), measured after 15 min in the presence of TPA. Results are expressed as a percentage of ^{32}P labelling in the absence of TPA, measured 15 min after an initial 5 min incubation. Results are mean ± S.E. from three separate experiments performed in duplicate.

by a factor of 10^5. Relevant control incubations were included and showed that DMSO alone, at a $1/10^5$ dilution, had no significant effect on basal histamine or LDH release (data not shown). Katakami et al. [11] also observed histamine release from rat mast cells due to the action of TPA alone, but did not detect any significant release of LDH. The reason for the discrepancy between our observations and those of Katakami et al. may reflect differences in preparation and handling of cells, or strain differences between animals used.

Effects of TPA on phospholipid metabolism in mast cells

TPA caused a dose-dependent increase in $^{32}PO_4^{3-}$ incorporation into PC and PA, but did not influence the labelling of PI (Fig. 2). It is evident that the effect of TPA on phospholipid metabolism in mast cells is potent, since a large measurable response was observed in spite of its possible cytotoxic effects.

In a kinetic study (Fig. 3), TPA (10 ng/ml) caused a gradual increase in the labelling of PA and PC over a period of 15 min. In contrast to this observation, it has been shown that histamine-releasing stimuli such as anti-IgE [13] and compound 48/80 [21] caused a rapid increase in PA labelling occurring within 3 s of stimulation. The kinetics of the rise in PC labelling induced by TPA was comparable to that observed when mast cells were stimulated by histamine-releasing stimuli.

The effect of TPA on histamine release and phospholipid metabolism in mast cells stimulated with histamine-releasing secretagogues

The synergistic action of TPA in promoting histamine release from mast cells stimulated with immunologic stimuli or sub-optimal doses of A23187 has been reported previously [11,12]. Heiman and Crews [12] also demonstrated that this property of TPA was selective, since histamine

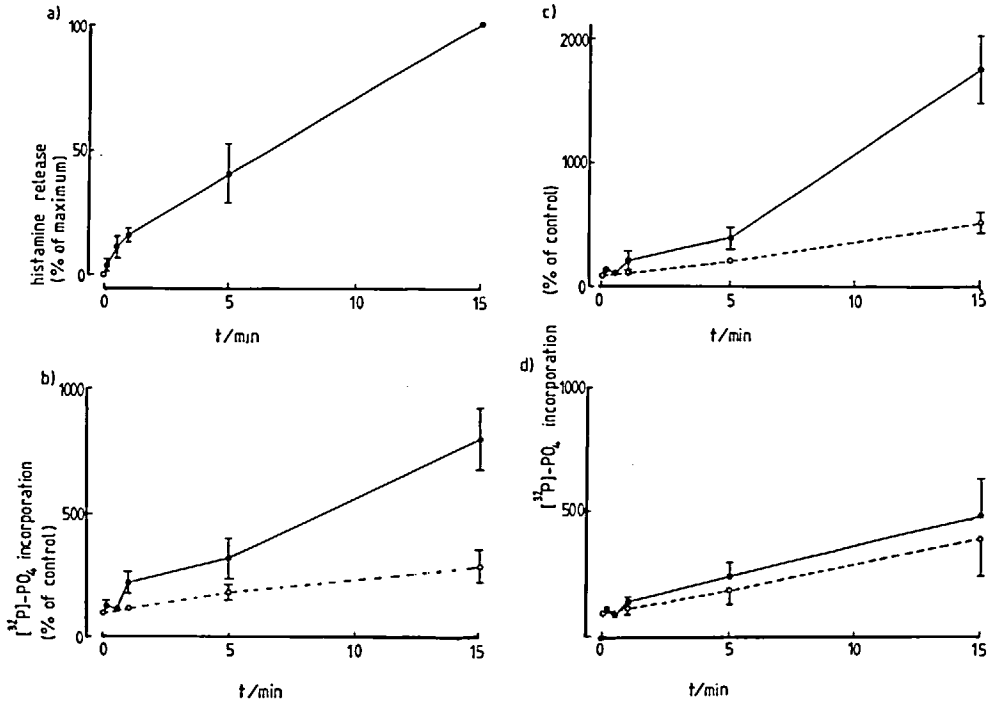

Fig. 3. Kinetic effects of TPA (10 ng/ml) on histamine release (a) and $^{32}PO_4^{3-}$ incorporation into PA (b) PC (c) and PI (d) in the presence (●) and absence (○) of TPA. Phospholipid labelling was expressed as a percentage of labelling at $t = 0$. Results are means ± range from two separate experiments performed in duplicate.

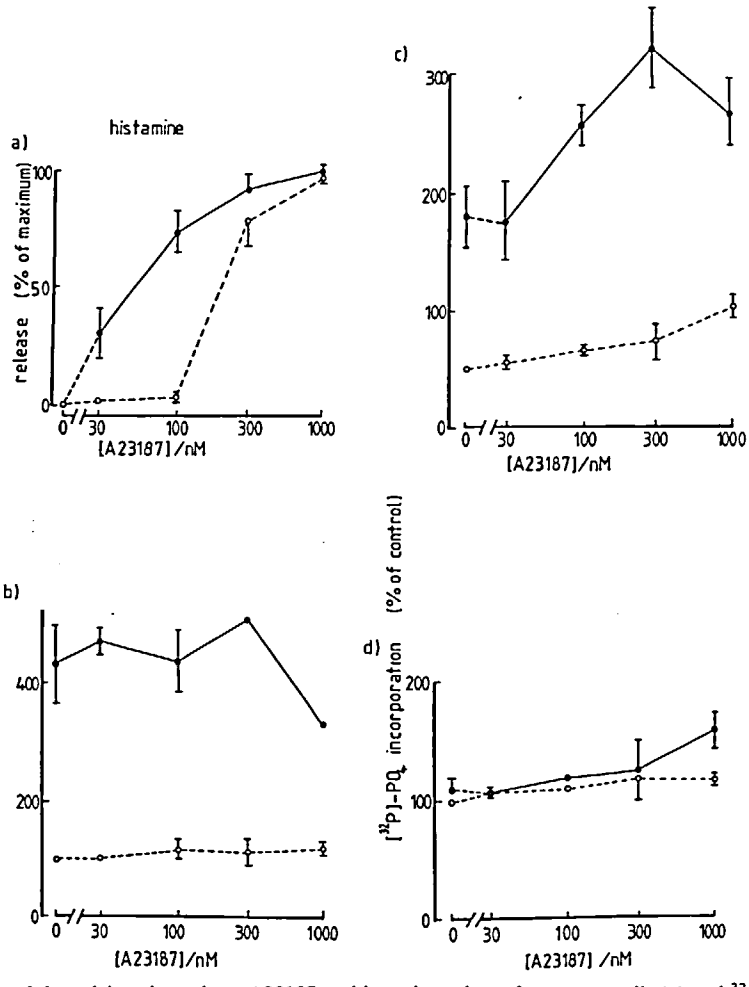

Fig. 4. Dose-related effects of the calcium ionophore A23187 on histamine release from mast cells (a) and $^{32}PO_4^{3-}$ incorporation into PC (b) PA (c) and PI (d) in the presence (●) and absence (○) of TPA (10 ng/ml). Results are expressed as a percentage of ^{32}P labelling in the absence of stimulus and TPA. Mean ± range from two separate experiments performed in duplicate are shown.

TABLE I

THE EFFECT OF TPA ON $^{32}PO_4^{3-}$ LABELLING OF PHOSPHOLIPIDS AND HISTAMINE SECRETION FROM MAST CELLS, STIMULATED WITH ANTI-IgE IN THE PRESENCE OF PS

A final concentration of 10 ng/ml TPA, 20 μg/ml PS and a dilution of anti-IgE of 1/200 was used. Labelling of phospholipids was determined as described in Materials and Methods. Values for $^{32}PO_4^{3-}$ incorporation are expressed as a percentage of labelling in the absence of both anti-IgE and TPA. However, where PS was to be included in incubations with stimulated cells it was also included in the control incubation. The synergy ratio was calculated from raw data from each experiment from the following formula: Synergy ratio = (dpm in secretagogue and TPA-treated sample) − (background dpm)/(dpm in secretagogue treated sample) + (dpm in TPA treated sample) − (2 × background dpm). Maximal histamine release, expressed as a percentage of total cellular histamine, was 35.4 ± 6.9%: results are mean ± S.E. from three separate experiments.

Additions	$^{32}PO_4^{3-}$ incorporation (% control)			Histamine release (% of maximum)
	PI	PC	PA	
TPA	142 ± 9.5	453 ± 58	331 ± 27	
anti-IgE	272 ± 43	242 ± 29	185 ± 20	64.9 ± 9.1
anti-IgE + TPA	244 ± 52	646 ± 139	607 ± 149	100
Synergy ratio	0.55 ± 0.1	1.13 ± 0.1	1.75 ± 0.18	

TABLE II

THE EFFECT OF TPA ON $^{32}PO_4^{3-}$ LABELLING OF PHOSPHOLIPIDS AND HISTAMINE SECRETION FROM MAST CELLS STIMULATED WITH COMPOUND 48/80

A final concentration of 10 ng/ml TPA and 0.7 μg/ml 48/80 was used. Labelling of phospholipids was determined as described in Materials and Methods. Values for $^{32}PO_4^{3-}$ labelling are expressed as a percentage of labelling in the absence of both TPA and compound 48/80. The synergy ratio was calculated as described in Table I. Maximal histamine release, expressed as a percentage of total cellular histamine, was 63.9±1.1%: results are mean±S.E. from three separate experiments.

Additions	$^{32}PO_4^{3-}$ incorporation (% control)			Histamine release (% of maximum)
	PI	PC	PA	
TPA	141 ±14	428 ±48	248 ± 32	-
48/80	269 ±16	326 ±59	212 ± 27	97.5±2.5
48/80+TPA	224 ±31	548 ±12	699 ±162	99.2±0.75
Synergy ratio	0.58± 0.13	0.97± 0.13	1.6± 0.15	-

release induced by compound 48/80 and polypeptide stimuli (represented by somatostatin) was not potentiated by TPA. In the present study, these findings have been confirmed. It was observed that TPA (10 ng/ml) potentiated histamine release induced by anti-IgE and A23187 (Fig. 4 and Table I), but not compound 48/80 (Table II). Some of the changes in phospholipid metabolism observed in cells stimulated by these various secretagogues in the presence of TPA, did not, however, correlate with the observed changes in histamine release.

TPA synergistically enhanced histamine release from mast cells stimulated with a (normally) suboptimal dose of the calcium ionophore A23187 (Fig. 4). Under normal conditions, A23187 did not increase $^{32}O_4^{3-}$ incorporation into PC or PI, and the increase in PA labelling reached 202% of control labelling at a maximal (1 μM) dose of A23187. When TPA (10 ng/ml) was included in incubations, there was no change in PI labelling or any change in PC labelling that could be related to the effects of A23187. The increase in PC labelling observed arose only as a result of the effects of TPA. In contrast, the labelling of PA was synergistically increased in the presence of TPA in a manner which suggested that it was modulated in a dose-dependent fashion by A23187.

The reduction in PC and PA labelling at higher doses of A23187 may have been due to decreased cell viability resulting from the combination of the known cytotoxic effect of TPA and possible cytotoxicity of supraoptimal doses of A23187.

In Table I, the effect of TPA on phospholipid metabolism and histamine release from mast cells stimulated with anti-IgE (1/200 dilution) in the presence of PS (20 μg/ml) is shown. TPA (10 ng/ml) synergistically enhanced the release of histamine and $^{32}PO_4^{3-}$ incorporation into PA but did not alter the labelling of PC beyond that which was an additive effect. Labelling of PI induced by a combination of anti-IgE and TPA was inhibited with respect to the sum of the increases in PI labelling when these two compounds are added separately to mast cells. In mast cells stimulated by compound 48/80 in the presence of TPA, a similar pattern of changes in phospholipid labelling was observed, but histamine release was not enhanced by TPA, even though a suboptimal dose of compound 48/80 was used (as can be seen in Table II).

Discussion

The aim of the studies described here was to investigate whether the effects of phorbol esters upon histamine release, stimulated by a variety of secretagogues were paralleled by corresponding changes in phospholipid turnover. The results from studies in which TPA was employed alone suggest that the mechanism by which TPA stimulates phospholipid turnover may be different from that observed when histamine-releasing secretagogues were used, since, unlike secretagogues such as compound 48/80 and anti-IgE, which have been shown to stimulate PI turnover and cause a rapid

increase in PA turnover [13,14,30], TPA causes a slow increase in PA and PC turnover, but does not affect PI turnover. This may indicate that these events in particular are more closely associated with the secretory response in mast cells.

Our studies indicate that TPA has cytotoxic properties, as exemplified by its ability to release LDH from mast cells. It is, therefore, not possible to determine whether TPA acts as a histamine-releasing agent in its own right, since the observed histamine release probably occurred due to leakage across damaged plasma membranes. However, over a short time course, the cytotoxic effects of TPA do not seem to interfere with its ability to stimulate phospholipid turnover or co-stimulate histamine release.

The selectivity of TPA in stimulating phospholipid turnover in mast cells has also been observed in other tissues, all of which display increased labelling of PC [30,3]. The effect of TPA upon the labelling of other phospholipid species is more variable between different tissues, although the functional significance of these variations is not established.

In studies on other tissues it has been shown that TPA increases PC synthesis by directly stimulating the main regulatory enzyme for PC synthesis; cholinephosphate cytidylyltransferase (EC 2.7.7.15) [22] and may also increase breakdown of PC by a phospholipase C-like activity [3,4].

It is highly probable that the CDP-choline pathway for PC biosynthesis is involved in the increase in phospholipid turnover observed in mast cells, since this pathway represents the only route by which labelled phosphate can become incorporated into PC.

In this study we have shown that in mast cells stimulated to release histamine by various secretagogues in the presence of TPA the relationship between the effects on histamine release and phospholipid metabolism are variable, depending upon the nature of the secretagogue.

The combined effects of A23187 and TPA on histamine release from mast cells has been reported previously [11,12] and supports our findings that TPA caused a decrease in EC_{50} for A23187 from 200 nM to 50 nM. As reported previously, A23187 only minimally stimulates turnover of phospholipids as compared with other stimuli [13], but the dramatic effect of TPA upon histamine release is not paralleled by similar dose-related changes in phospholipid turnover, except for the increase in sensitivity of PA turnover to A23187 concentration in the presence of TPA. The lack of PI turnover observed here may indicate that TPA and A23187 stimulate secretion and increase turnover of PA without influencing metabolism of inositol phospholipids.

Studies using anti-IgE and compound 48/80 to stimulate mast cells in the presence of TPA show changes in phospholipid turnover which are remarkably similar and to the findings reported here using A23187. All three stimuli in the presence of TPA caused a synergistic increase in PA turnover, had no effect on PC turnover and, in the case of anti-IgE and compound 48/80, actually inhibited PI turnover. Studies by other workers have also shown this phenomenon, as well as inhibition of the hydrolysis of phosphatidylinositol 4,5-bisphosphate by TPA [26–28]. It has also been shown, in other tissues, that this inhibition is associated with protein kinase C activity [29].

Our observations, reported here, and those of others [12] seem to indicate that protein kinase C may play a role in the secretory mechanism in mast cells, however, further definitive experimentation is needed to establish this as fact. We have shown here that the phorbol ester TPA causes changes in mast cell phospholipid turnover independently of histamine release and, when TPA is used to potentiate histamine release, there is no corresponding change in phospholipid turnover.

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